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Regulation of Protein Synthesis Directed by Coliphage MS2 RNA

I. Phage Protein and RNA Synthesis in Cells Infected with Suppressible Mutants

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In actinomycin-treated *Escherichia coli* infected with coliphage MS2 three phage-specific proteins have been detected after acrylamide gel electrophoresis. These proteins have been identified by the use of suppressible mutants of MS2 as the phage coat protein, "maturation" or assembly protein and RNA synthetase. In MS2-infected cells RNA synthetase appeared before the other two proteins were detectable. All three proteins then increased, but the synthesis of maturation protein and RNA synthetase rapidly declined between 30 and 40 minutes post-infection, whereas the synthesis of coat protein continued at a high rate throughout the infectious cycle. The synthesis of phage RNA paralleled that of the RNA synthetase and maturation protein. Non-permissive cells infected with suppressible RNA synthetase mutants made no detectable phage proteins. Cells infected with maturation protein mutants showed a normal pattern of coat protein and RNA synthetase formation. Infection with a coat protein mutant resulted in a greater than normal rate of formation of RNA synthetase and maturation protein relative to the amount of phage RNA, and a higher percentage of each of these proteins was made late in infection compared to the situation in MS2-infected cells. However, the increase of these proteins was not co-ordinate: RNA synthetase formation increased more than the synthesis of maturation protein. In cells doubly infected with a maturation protein mutant and a coat protein mutant the derepression of synthetase and maturation protein seen in cells singly infected with the coat mutant was largely reversed. Our major conclusion is that the coat protein of MS2 serves as a repressor of the synthesis of maturation protein and RNA synthetase.

1. Introduction

During the replication of viruses some of the virus-specified proteins synthesized in infected cells serve as enzymes, whereas others are structural components of the virus particle. In cells infected with certain DNA viruses, such as the T-even bacteriophages, it is well known that the major structural proteins are made in greater quantity than are other proteins and that specific viral proteins are synthesized at particular times during the replicative cycle (Cohen, 1961; Sarabhai, Stretton, Brenner & Bolle, 1964; Levinthal, Hosoda & Shub, 1967). Although the detailed

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mechanism of this regulation is not known, it appears to be due in part to changes in the rates of synthesis of different messenger RNA molecules (Bolle, Epstein, Salser & Geiduschek, 1968). In the case of small, single-stranded RNA viruses, such as coliphage MS2 or poliovirus, it has been shown that the synthesis of viral proteins is also regulated in such a way that proteins required in small amount are present in infected cells in much lower concentration than the major structural protein(s) of the virus, and (in the case of MS2) that the rates of synthesis of different viral proteins vary during virus development (Summers, Maizell & Darnell, 1965; Oeschger & Nathans, 1966; Nathans, Oeschger, Eggen & Shimura, 1966; Viñuela, Algranati & Ochoa, 1967). Since the RNA of such viruses serves directly as a polygenic template for protein synthesis (Nathans, Notani, Schwartz & Zinder, 1962; Ohtaka & Spiegelman, 1963; Capecchi, 1966), this regulation is likely to occur at the level of protein synthesis.

The use of an RNA coliphage such as MS2 to study regulation of protein synthesis at the translation step is particularly advantageous, first, because the RNA appears to code for only three proteins (Horiuchi, Lodish & Zinder, 1966; Gussin, 1966), an RNA synthetase, a maturation protein (also called A protein or "assembly" protein), and phage coat protein, each of which can be measured in actinomycin-treated, infected cells (Nathans *et al.*, 1966; Viñuela *et al.*, 1967); and second, because the RNA serves as a polygenic messenger in cell extracts. In this paper we present results of experiments in which the rate of synthesis of each of the three phage proteins has been measured in cells infected with MS2 or with suppressible mutants of MS2 having mutations in each of the three cistrons; in the following paper the results of *in vitro* experiments on regulation of phage protein synthesis will be presented. Our results show that the major manifestation of regulation in infected cells is the early decrease in the rate of synthesis of RNA synthetase and maturation protein, the two proteins required in small amount, in contrast to the continued rapid synthesis of the coat protein, the major structural protein of the virus. This decline in synthesis of the two minor phage proteins appears to be due to direct repression by the phage coat protein. Experiments along similar lines have also been reported by Viñuela *et al.*, 1968.

2. Materials and Methods

(a) *Bacteria and bacteriophages*

Escherichia coli strains S26 (*su*$^-$), S26Rle (*su-1*), and C600 (*su-2*) were obtained from N. D. Zinder; C3000 (*su*$^-$) from R. L. Sinsheimer; and A19 from W. Gilbert. A mutant of C3000 (C3000-42) requiring lysine, arginine, isoleucine and valine was isolated as previously described (Oeschger & Nathans, 1966). Bacteriophage MS2 was obtained originally from R. L. Sinsheimer. Suppressible mutants were isolated from stocks mutagenized with nitrous acid (Zinder & Cooper, 1964) by testing single plaques on *E. coli* S26R1e and S26 as described by Gussin (1966). In order to exclude temperature-sensitive missense mutants, each suppressible mutant was repeatedly cloned at 42°C on *E. coli* C600 or, in the case of coat protein mutants, on *E. coli* S26R1e. Phage stocks were prepared by inoculating 10 ml. of a culture of S26Re (growing at 37°C in modified TPG medium (Oeschger & Nathans, 1966) at a cell density of 5×10^7/ml.) with a stab from a single plaque of mutant phage growing on a TPG plate. After 2·5 hr the culture was lysed as described previously (Shimura, Moses & Nathans, 1965). The mutant stocks used in the experiments to be described had about 10^{11} plaque forming units/ml. and reversion frequencies of 0·02 to 0·5%.

(b) *Detection of defective phage particles*

Mutants were grown in *E. coli* S26 and purified by ammonium sulfate precipitation and CsCl centrifugation as previously described (Shimura *et al.*, 1965). For the *detection of maturation protein in phage particles*, [^{14}C]amino acid-labeled phage was prepared by

infecting *E. coli* A19, previously treated with EDTA and actinomycin (Richelson & Nathans, 1967), with the appropriate mutant, and adding a mixture of [^{14}C]amino acids 15 min later. At 60 min after infection 0·01 vol. of 10% Casamino acids (Difco) was added, and at 75 min the cells were chilled, washed and sonicated. After centrifugation of the sonicate at 10,000 *g* for 5 min, ^{14}C-labeled phage particles were isolated by CsCl centrifugation followed by pelleting through 10% sucrose. The phage pellets were then solubilized and electrophoresed as described below.

(c) *Phage RNA and protein synthesis*

These were measured in actinomycin-treated *E. coli* C3000-42, as described earlier (Oeschger & Nathans, 1966). In a typical experiment bacteria sensitized with EDTA were shaken at 37°C at a concentration of 4×10^8 cells/ml. in modified TPG medium, containing 6 or 7 μg actinomycin/ml., 8×10^{-4} mM-lysine, arginine, isoleucine and valine and 0·03 mM-uridine. After 5 min the cells were infected at a multiplicity of 4 plaque forming units per cell. At the appropriate time a portion of the culture was transferred to tubes containing either [^{3}H]uridine (5 μC/ml., 1 C/m-mole) or a mixture of [^{14}C]lysine, arginine, isoleucine, and valine (a total of 0·5 μC/ml., each amino acid about 200 μC/μmole). At the end of the labeling period, 0·1 ml. portions of the culture were applied to Whatman 3 MM filter paper discs and washed and counted as described earlier (Oeschger & Nathans, 1966).

(d) *Analysis of specific phage proteins*

Cells labeled with [^{14}C]amino acids were sedimented at 10,000 *g* for 5 min and the medium discarded. Over 99% of the radioactive protein was in the sediment. The cell pellet was stored frozen at −65°C until analyzed. To solubilize phage proteins the cells were suspended in a small volume (0·05 to 0·10 ml.) of 1% sodium dodecyl sulfate–0·05 M-EDTA–0·05 M-mercaptoethanol and incubated at 36°C for 60 min. The lysate was then dialyzed against 0·1% sodium dodecyl sulfate–0·01 M-mercaptoethanol and electrophoresed in 6-cm columns of 7·5% polyacrylamide gel containing 0·1% sodium dodecyl sulfate, 0·5 M-urea and 0·05 M-sodium phosphate of pH 7·2 (modified from Maizell, 1966). After electrophoresis for 3·5 hr at 5 mA per tube, the gels were transferred to 5% trichloroacetic acid and left overnight at 4°C. They were then treated with 5% trichloroacetic acid at 75°C for 15 min and washed 6 times with 7·5% acetic acid. The gels were sliced longitudinally and the slices dried for radioautography by the method of Fairbanks, Levinthal & Reeder (1965). To measure phage proteins, the dried gel slices were exposed to X-ray film (Kodak No Screen) for sufficient times to give film blackening proportional to radioactivity for each protein peak,

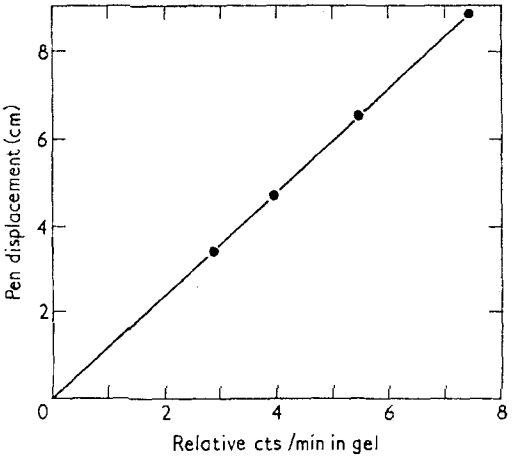

FIG. 1. Proportionality between the height of the densitometer tracing of the radioautogram and the amount of [^{14}C]protein present in the polyacrylamide gel. Different amounts of [^{14}C]protein were added to acrylamide solutions prior to polymerization, and the resulting gels were processed for radioautography as described in Materials and Methods. The dried gels were exposed to X-ray film for 12 days. One unit of radioactivity corresponds to 800 cts/min in a 6-cm gel column.

as determined by a series of standards. The radioautogram was then scanned on a Joyce–Loebl microdensitometer and the area of each protein peak measured with a planimeter. Figure 1 shows the proportionality between the height of the tracing and the amount of radioactive protein in the gel with a series of gels containing known amounts of [^{14}C]protein. In addition it was shown that the degree of blackening was proportional to exposure time. In order to convert the area of a protein peak to cts/min/ml. of original culture, the area of the tracing of the entire radioautogram was set equivalent to the total radioactivity applied to the gel and the cts/min in each protein peak calculated. Each value was then expressed as cts/min/ml. of cell culture.

(e) *Materials*

These were obtained from sources given previously (Oeschger & Nathans, 1966; Nathans *et al.*, 1966).

3. Results

(a) *Properties of amber mutants*

Five suppressible mutants of MS2, isolated as described in Materials and Methods, were chosen for the experiments reported in this paper. As shown below, two of these mutants have mutations in the RNA synthetase gene, two have mutations in the maturation protein gene, and one has a mutation in the coat protein gene. All were characterized by their effects on the growth of *su*$^-$ cells (S26), efficiency of plating on *su-1* (S26R1e) and *su-2* (C600) suppressor strains, phage RNA and protein synthesis in actinomycin-treated, non-permissive host bacteria, and electrophoresis of phage proteins present in infected cells. In the case of suspected maturation protein mutants, CsCl gradient centrifugation of phage particles was used to detect defective particles; these particles were also analyzed for the presence of maturation protein.

The effect of each mutant on the growth of *su*$^-$ cells is presented in Table 1. *am9* and *am13* had no effect on growth, *am12b* caused cessation of growth but no lysis; and *am5* and *am8b* caused normal lysis. Based on Zinder & Cooper's observations with f2 mutants (Zinder & Cooper, 1964), these growth patterns tentatively localized the mutations in *am9* and *am13* to the RNA synthetase gene, *am12b* to the coat protein gene and *am5* and *am8b* to the maturation protein gene.

The relative efficiency of plating of the above mutants on S26R1e (*su-1*) and C600 (*su-2*) revealed that only *am12b* had a lower efficiency on the *su-2* than on the *su-1* suppressor strain (Table 1). As shown by Zinder & Cooper (1964) and by Gussin (1966),

TABLE 1

Mutant	Effect on growth of *E. coli*	Efficiency of plating *su-2*/*su-1*	Phage RNA synthesis	Phage proteins			Classification of mutant
				Synthetase	Maturation protein	Coat	
am9	none	0·9	none	−	−	−	RNA synthetase
am13	none	1·0	none	−	−	−	RNA synthetase
am5	lysis	1·4	normal	+	−	+	Maturation protein
am8b	lysis	1·1	decreased	+	−†	+	Maturation protein
am12b	inhibition	0·01	decreased	+	+	−	Coat protein

† A new protein is present with an electrophoretic mobility greater than that of the normal maturation protein.

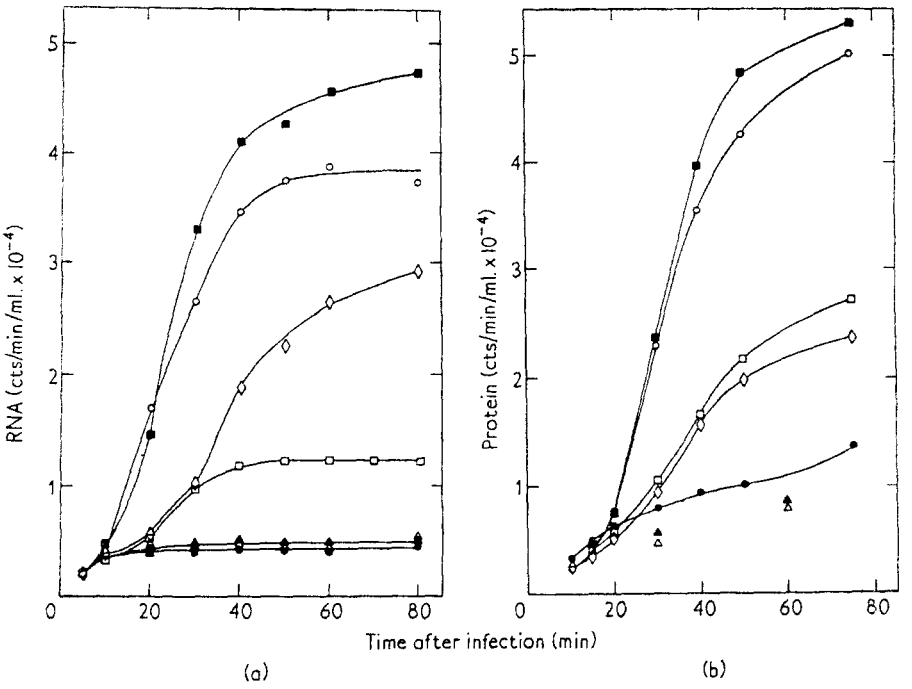

FIG. 2. RNA and protein synthesis in actinomycin-treated *E. coli* C3000-42 infected with various mutants. Portions of a single batch of actinomycin-treated cells were infected with various mutants or MS2 at a multiplicity of 4. For measurement of RNA synthesis, [^{3}H]uridine was added 5 min after infection and its incorporation into cold trichloroacetic acid-precipitable radioactive material was determined at intervals after infection. For measurement of protein synthesis, portions of the cells were added to a mixture of [^{14}C]amino acids at various times and the amount of hot trichloroacetic acid-precipitable radioactivity measured after 5, 10 or 25 min exposure to the [^{14}C]amino acid mixture. The results are plotted as the cumulative cts/min in protein.

(a) RNA synthesis; (b) protein synthesis. —●—●—, unifected cells; —○—○—, MS2-infected cells; —■—■—, *am5*-infected cells; —□—□—, *am8b*-infected cells; —◇—◇—, *am12b*-infected cells; —△—△—, *am9*-infected cells; —▲—▲—, *am13*-infected cells.

coat protein mutants yield no plaques or barely visible plaques on the *su-2* suppressor strain since this strain inserts glutamine at the nonsense codon with very low frequency. Therefore, this result also suggests that only *am12b* is a coat protein mutant.

Measurement of phage RNA and total phage protein synthesis was carried out in actinomycin-treated *su*$^-$ bacteria (C3000-42) infected with wild type MS2 or with each of the various mutants. As seen in Figure 2, the results with *am5* were very similar to those with MS2; *am8b* and *am12b* gave reduced phage RNA and protein synthesis; and cells infected with *am9* or *am13* were indistinguishable from uninfected cells. These results are consistent with the conclusion that only *am9* and *am13* have nonsense mutations in the RNA synthetase gene.

Electrophoresis of phage proteins found in actinomycin-treated, *su*$^-$ cells after infection with each of the mutants gave the results shown in Figure 3. Cells infected with wild type MS2 (Fig. 3(b)) showed three reproducible phage proteins, labeled 1, 2 and 3. As described previously (Nathans *et al.*, 1966), peak 3 is the major coat protein of the phage, peak 2 is the maturation protein and peak 1, by inference, is the phage RNA synthetase. In addition, other smaller peaks are occasionally seen, but because of their inconstant occurrence these are thought to be derived from the other phage proteins.

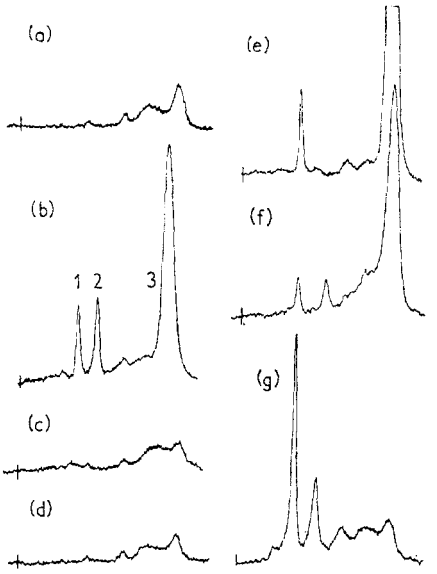

FIG. 3. Microdensitometer tracings of radioautograms of electrophoresis gels containing ^{14}C-labeled phage proteins from infected cells. Phage proteins were labeled with a mixture of [^{14}C]amino acids as described in Materials and Methods.

(a) Uninfected cells; (b) MS2-infected cells; (c) *am9*-infected cells; (d) *am13*-infected cells; (e) *am5*-infected cells; (f) *am8b*-infected cells; (g) *am12b*-infected cells.

The molecular weights of the peak 1 and peak 2 proteins can be estimated by their electrophoretic mobilities as described by Schapiro, Viñuela & Maizell (1967). The peak 1 protein has an electrophoretic mobility between that of bovine serum albumin and the heavy chain of γ-globulin and has an estimated molecular weight of 60,000. Peak 2 has a mobility slightly greater than that of dissociated *E. coli* alkaline phosphatase and has an estimated molecular weight of about 35,000, in agreement with the findings of Argetsinger-Steitz (1968).

Cells infected with *am9* (Fig. 3(c)) or *am13* (Fig. 3(d)) showed no phage-specific proteins, a result consistent with a defect in RNA synthesis. (However, in Su^- cell-free extracts supplemented with *am9* or *am13* RNA, synthesis of a protein corresponding in mobility to the peak 1 protein was markedly stimulated by the addition of Su^+ transfer RNA (Schmickel, Eggen & Nathans, manuscript in preparation). This result confirms the inference that peak 1 protein is the RNA synthetase.) Cells infected with *am12b* (Fig. 3(g)) showed excess RNA synthetase, normal or slightly elevated levels of maturation protein and no coat protein. Since temperature-sensitive mutants involving the synthetase gene may also yield high levels of synthetase protein at low temperature (Nathans, unpublished observations), cells infected with a revertant of *am12b* selected on *E. coli* S26 were also analyzed. The electrophoretic pattern in this case was similar to that observed with wild type MS2 (Fig. 4), indicating that the high level of synthetase seen in *am12b*-infected cells was not due to a second, missense synthetase mutation. Cells infected with *am5* (Fig. 3(e)) showed no maturation protein, but essentially normal levels of synthetase and coat protein; and cells infected with *am8b* (Fig. 3(f)) showed somewhat reduced synthetase and coat protein but instead of the maturation protein there was a new protein peak with increased electrophoretic

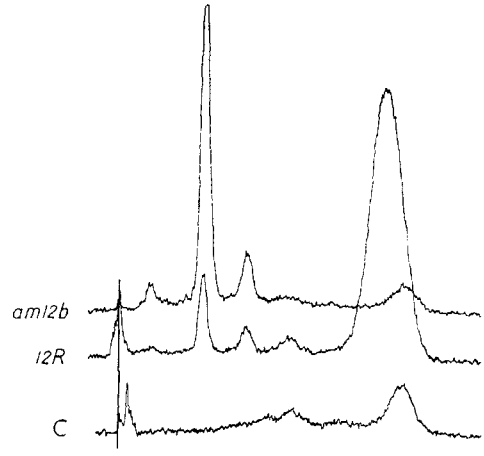

FIG. 4. Tracings of radioautograms of gels containing phage proteins from cells infected with *am12b* (top), a revertant of *am12b* (*12R*, middle tracing) or from uninfected cells (bottom).

mobility. This peak probably represents a polypeptide fragment of the maturation protein. The low levels of phage protein seen in cells infected with *am8b* correlates with the reduced level of phage RNA synthesized (Fig. 2). Revertants of this mutant are not significantly different in these respects from the mutant, suggesting that *am8b* is not polar. The cause of the decreased rate of RNA synthesis in *am8b*-infected cells has not been established; one possibility is that this phage harbors a second, missense mutation in the synthetase gene.

RNA-deficient particles of the type described by Lodish, Horiuchi & Zinder (1965); Heisenberg (1966); and Argetsinger & Gussin (1966), were observed by CsCl gradient centrifugation of lysates of *E. coli* S26 infected with *am5* or *am8b* as well as with three other mutants not further described here which failed to produce maturation protein in infected cells. In contrast with wild type MS2 particles, neither *am5* nor *am8b* particles contained detectable maturation protein (Fig. 5), confirming previous

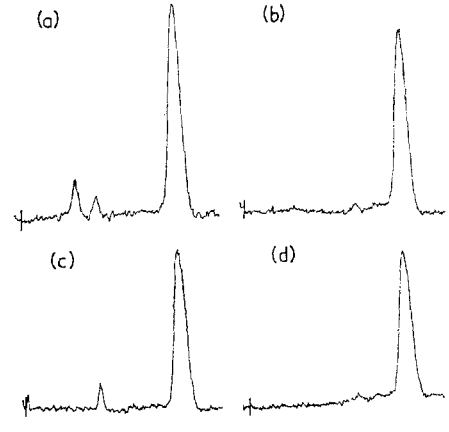

FIG. 5. Microdensitometer tracings of [^{14}C]proteins from bacteriophage particles isolated from actinomycin-treated *E. coli* A19 infected with *am5* (b), MS2 (c) or *am8b* (d). The cells were labeled and the phage purified as described in Materials and Methods. Tracing (a) is an analysis of [^{14}C]phage proteins in MS2-infected cells.

reports that the maturation protein is a minor structural component of MS2 and related phages (Nathans *et al.*, 1966; Roberts & Argetsinger-Steitz, 1967; Argetsinger-Steitz, 1968).

From the series of experiments described above and summarized in Table 1 we conclude that *am9* and *am13* are RNA synthetase mutants; *am12b* is a coat protein mutant; *am5* and *am8b* are maturation protein mutants, with *am8b* having a more distal mutation than *am5*.

(b) *Rates of synthesis of phage proteins*

(i) *MS2-infected cells*

As shown previously, the rate of synthesis of MS2 coat protein in infected cells greatly exceeds the rate of synthesis of non-coat proteins, and the synthesis of non-coat proteins levels off sooner than that of the coat protein (Oeschger & Nathans, 1966; Nathans *et al.*, 1966; Viñuela *et al.*, 1967). When the synthesis of the three phage proteins was measured by pulse-labeling actinomycin-treated, infected *E. coli* C3000-42 with [^{14}C]amino acids, the first protein to be detected was the peak 1 protein or RNA synthetase, which appears between 5 and 15 minutes after infection (Figs 6(a) and 7). Several minutes later the maturation protein first appears, and at about the same time coat protein starts to increase. (Any coat protein synthesized during the first 15 minutes after infection may be obscured, however, by the protein formed in

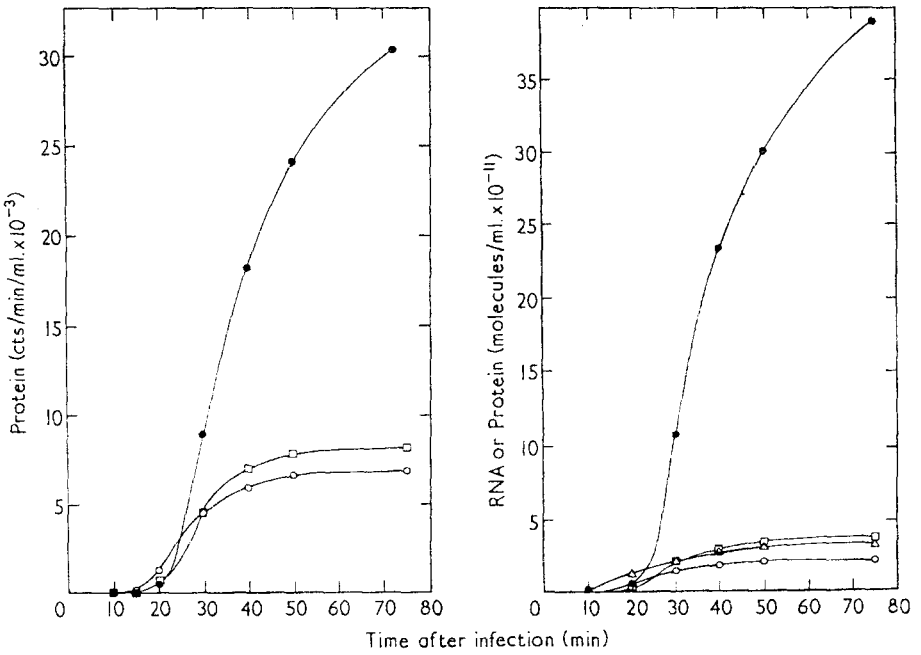

FIG. 6. Kinetics of individual phage protein synthesis.

A culture of actinomycin-treated *E. coli* C3000-42 was infected with MS2 at a multiplicity of 4 and pulse-labeled with a mixture of [^{14}C]amino acids or continuously labeled with [^{3}H]uridine. The amount of each phage protein was determined as described in Materials and Methods; the values plotted are cumulative cts/min in each phage protein peak. A control consisting of pulse-labeled uninfected cells was similarly analyzed and the amount of radioactivity present in the protein peak which coincided with the phage coat protein was subtracted. On the left (a) the results are plotted as amount of radioactive protein; and on the right (b) as number of molecules or, in the case of RNA, as phage equivalents. —○—○—, Synthetase; —□—□—, maturation protein; —●—●—, coat protein ;—△—△—, RNA.

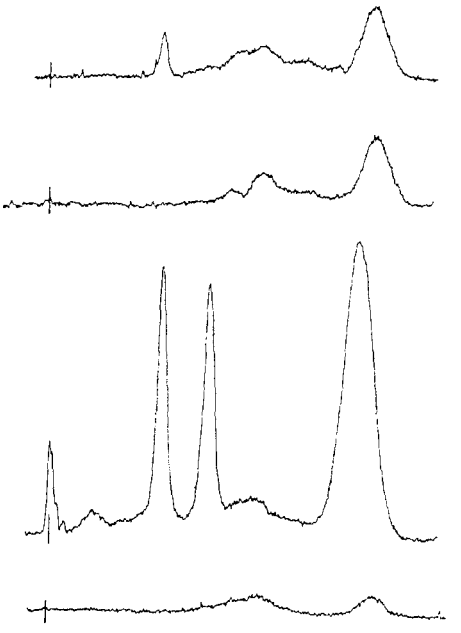

FIG. 7. Microdensitometer tracings of radioautograms of gels containing pulse-labeled early phage proteins. A culture of actinomycin-treated *E. coli* C3000-42 was divided into two portions, one of which was infected with MS2 at a multiplicity of 4. The cells were then pulse-labeled with a mixture of [^{14}C]amino acids and the proteins analyzed as described in Materials and Methods.
Top tracing: infected cells labeled from 5 to 15 min after infection.
Second tracing: uninfected cells labeled from 5 to 15 min.
Third tracing: infected cells labeled from 15 to 25 min after infection.
Bottom tracing: uninfected cells labeled from 15 to 25 min.

uninfected cells, which has a similar electrophoretic mobility.) The most striking aspect of the kinetics of protein synthesis is the early decline in the rate of synthesis of both the synthetase and the maturation protein, while coat protein synthesis continues at a high rate. Of note is the reproducible observation that synthetase formation declines faster than the formation of maturation protein. In Figure 6(b) the amount of each protein is plotted as the number of molecules per ml. of culture and is compared with the phage equivalents of RNA. Although the average burst size of MS2 in actinomycin-treated cells is only about 20% of normal (Oeschger & Nathans, 1966), it is nevertheless of interest to estimate the relative numbers of phage protein molecules under these conditions. These values were calculated from the specific activities of radioactive precursors, assuming molecular weights for maturation protein and synthetase of 35,000 and 60,000, respectively (see above), and assuming that the amino acids used for labeling were as abundant in synthetase and maturation protein as in phage coat protein (Lin, Tsung & Fraenkel-Conrat, 1967). As seen in the Figure, the number of coat protein molecules synthesized by 75 minutes post-infection is about 10 to 20 times that of each of the other two proteins. In agreement with our prior findings (Oeschger & Nathans, 1966), the phage equivalents of RNA are about ten times the phage equivalents of coat protein. Also of note is the ratio of coat protein molecules to phage equivalents of RNA when synthetase formation has nearly ceased: 5·3 at 30 minutes post-infection and 8·5 at 40 minutes. This is comparable to the molar

ratio of coat protein to phage RNA required for nearly complete inhibition of synthetase formation in cell-free extracts (Eggen & Nathans, 1969).

(ii) *Mutant-infected cells*

Rates of synthesis of individual phage proteins were also measured in Su$^-$ cells infected with each of the five amber mutants described above. In the case of *am9* and *am13* (RNA synthetase mutants), no proteins were detected throughout the 60 minutes

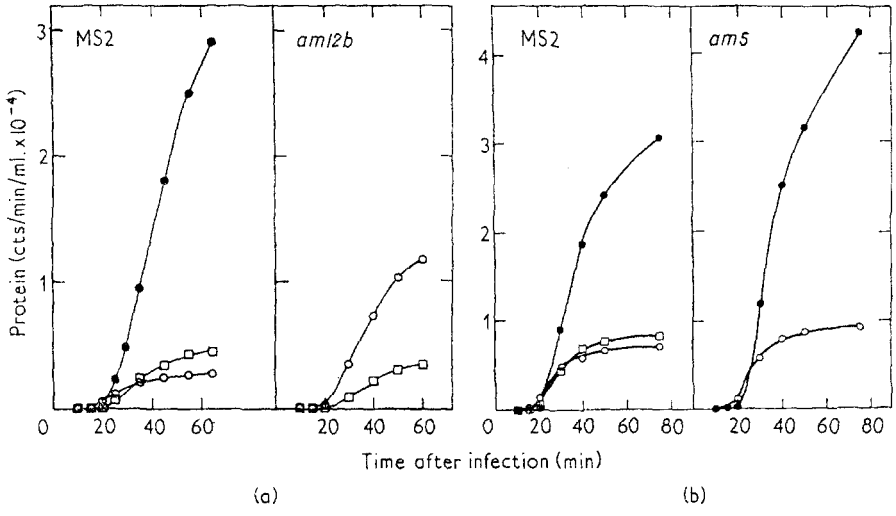

FIG. 8. Kinetics of individual phage protein synthesis in cells infected with mutants of MS2. A culture of actinomycin-treated *E. coli* C3000-42 was divided into three portions; one portion was not infected and each of the others was infected with MS2 or a mutant phage at a multiplicity of 4. Beginning 5 min after infection, the cultures were pulse-labeled with a mixture of [^{14}C]amino acids, as described in Materials and Methods, and the amount of each phage protein determined after electrophoresis and radioautography. The results are presented as cumulative cts/min in each protein after subtraction of the radioactive protein found in uninfected cells. (a) Results of an experiment in which MS2-infected cells are compared with *am12b*-infected cells. (b) Results of a second experiment in which MS2-infected cells are compared with *am5*-infected cells. —○—○—, Synthetase; —□—□—, maturation protein; —●—●—, coat protein.

post-infection (Fig. 3). The results with a coat protein mutant (*am12b*) and a maturation protein mutant (*am5*) are shown in Figure 8; in each case the results are compared with MS2-infected cells. As seen in Figure 8, cells infected with *am12b* maintained for a longer time a high rate of synthesis of RNA synthetase when compared to cells infected with MS2. The rate of synthesis of the maturation protein is considerably lower than that of synthetase; however, the rate is sustained in parallel with that of the synthetase. Relative to the amount of phage RNA present in *am12b*-infected cells, the amount of synthetase varied in several different experiments from three to six times the normal level, and the amount of maturation protein varied from about 0·9 to 2 times that found in MS2-infected cells. It should be noted that more of the RNA in *am12b*-infected cells is in double-stranded form than is the case in MS2-infected cells (Richelson & Nathans, unpublished observations; see also Lodish & Zinder, 1966), and therefore a smaller fraction of the RNA in *am12b*-infected cells would be available for translation.

In contrast to these results with *am12b*-infected cells, *am5*-infected cells show an essentially normal pattern of synthetase shut-off and coat protein synthesis (Fig. 8).

Likewise, in *am8b*-infected cells, the patterns of synthesis of RNA synthetase, coat protein, and the presumed maturation protein fragment are all essentially normal (results not shown), although, as noted earlier, such cells have low phage RNA and protein synthesis. Thus the lack of a normal maturation protein in *am5*- or *am8b*-infected cells does not lead to derepression, whereas the lack of a normal coat protein in *am12b*-infected cells results in obvious derepression of synthetase formation, and probably also of maturation protein synthesis. It is clear, however, that in *am12b*-infected cells derepression of the synthetase and maturation protein is not co-ordinate.

(iii) *Double infection with* am5 *and* am12b

In order to exclude the possibility that the derepression observed in *am12b*-infected cells was a secondary effect of the nonsense mutation itself rather than the lack of normal coat protein, viral protein synthesis was measured in cells doubly infected with *am12b* and *am5*. This experiment was meant to determine whether the coat protein produced by the *am5* RNA represses the formation of proteins produced by *am12b* RNA. Multiplicities of infection were chosen in the range where total phage protein synthesis was proportional to multiplicity and where a high percentage of doubly-infected cells would result. Also, to reduce the labeling of proteins synthesized early, [^{14}C]amino acids were added 40 minutes after infection.

Measurements of the synthesis of phage proteins in cells infected with *am12b* or *am5* alone (at a multiplicity of 2) or with both mutants simultaneously (each at a multiplicity of 2) are presented in Figure 9 in the form of tracings of the radioautograms of gels in which the phage proteins were electrophoresed, and in Table 2 as the amount of each protein synthesized. As seen in Figure 9 and in Table 2, the derepression of synthetase formation evident in cells infected with *am12b* alone is partially reversed by simultaneous infection with *am5*. In regard to maturation protein, the results show that the synthesis of this protein in doubly-infected cells is also less than in cells infected with *am12b* alone. The fact that repression is not completely restored may be due to physical separation within the cell of *am12b* RNA and *am5* coat protein molecules and to those cells which are infected with *am12b* alone. Our interpretation of the results of this experiment is that the coat protein made on *am5* RNA represses the synthesis of RNA synthetase and of maturation protein by *am12b* RNA. Hence the

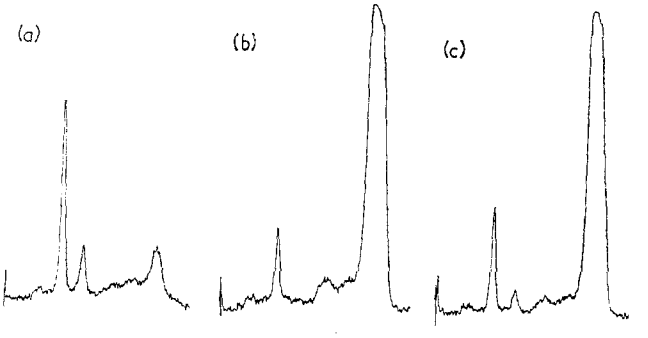

FIG. 9. Microdensitometer tracings of radioautograms of phage proteins from cells infected with *am12b* (a), *am5* (b) or *am5* plus *am12b* (c). A single batch of actinomycin-treated *E. coli* C3000-42 was infected with *am5* and/or *am12b* at a multiplicity of 2 and 40 min later [^{14}C]amino acids were added. At 60 min the cells were processed as described in Materials and Methods. (a) represents 0·9 ml. of infected cells; (b) 1·1 ml. and (c) 1·0 ml.

TABLE 2

Protein synthesis in cells infected with am5 *and* am12b

Phage	Synthetase	Maturation protein (cts/min/ml.)	Coat protein
Experiment 1			
am5	2310	0	44,400
am12b	8950	2490	0
am5 + *am12b*	4570	1050	51,600
Experiment 2			
am5	1650	0	39,300
am12b	6450	2290	0
am5 + *am12b*	3810	970	34,600

See legend to Fig. 9 for a description of the experiment. In experiment 1 each mutant was present at a multiplicity of 2. In experiment 2 each mutant was present at a multiplicity of 1·5.

derepression observed in *am12b*-infected cells appears to be due to the lack of normal coat protein.

4. Discussion

The principal conclusion of the experiments presented in this paper, taken together with those previously published (Lodish, Cooper & Zinder, 1964; Gussin, 1966; Viñuela *et al.*, 1968) is that the coat protein of MS2 regulates the synthesis of MS2 proteins in infected cells by selective inhibition of synthesis of the RNA synthetase and probably also of the maturation protein, the two proteins which are required in small amount for replication of the phage. The evidence that this selective inhibition is due to direct repression by the coat protein may be summarized as follows. In regard to the synthetase, measurements on extracts of infected bacteria by Lodish *et al.* (1964) and by Gussin (1966) showed that the activity of RNA synthetase in cells infected with non-polar coat protein mutants of f2 or R17 was greater than the activity in cells infected with wild type phage. With similar mutants of MS2 the results of Viñuela *et al.* (1968) and the present experiments demonstrate directly that this effect is due to increased synthesis of the RNA synthetase. The observation that cells infected simultaneously with a coat protein mutant and a maturation protein mutant produce partially repressed levels of RNA synthetase suggests that the increased levels in *am12b*-infected cells is due to lack of coat protein rather than to some other effect of the coat protein mutation. Moreover, *in vitro* observations demonstrate that MS2 coat protein can combine with MS2 RNA (Capecchi & Gussin, 1965; Sugiyama, Hebert & Hartman, 1967) and selectively inhibit the synthesis of non-coat proteins (Eggen & Nathans, 1967, 1969; Sugiyama & Nakada, 1967, 1968). A similar coat protein–RNA complex has also been detected in MS2-infected cells (Richelson & Nathans, 1967). It should be pointed out, however, that the results reported in this paper can be interpreted either on the basis of direct translational control or control of phage RNA synthesis by phage coat protein. As suggested recently by Robertson, Webster & Zinder (1968), if coat protein inhibits the synthesis of phage RNA beyond the coat protein cistron, and complete molecules of RNA were not available as

messengers late in infection, the pattern of phage protein synthesis reported here would be expected.

Although the role of coat protein in repression of RNA synthetase formation is rather clear, present evidence for its role as the primary regulator of maturation protein synthesis is not as direct. As with RNA synthetase, maturation protein synthesis sharply decreases after 35 to 40 minutes post-infection. However, the kinetics of formation of the two proteins are not identical. Maturation protein synthesis begins somewhat later and continues at a slow rate for a longer time so that characteristically the curve of maturation protein formation crosses that of RNA synthetase. By 60 minutes after infection, more radioactivity is generally found in the maturation protein than in the synthetase, even though, judging by their relative electrophoretic mobilities, the molecular weight of the synthetase exceeds that of the maturation protein. These observations and the non-co-ordinate change in the synthesis of maturation protein and synthetase in *am12b*-infected cells suggest that maturation protein synthesis may not be shut off by the same event which inhibits synthetase formation. Nonetheless, there is evidence that maturation protein synthesis is regulated by the coat protein. In cells infected with the coat protein mutant *am12b*, there was often an increase in maturation protein synthesis relative to phage RNA over that in MS2-infected cells, as was found also with the mutant studied by Viñuela *et al.* (1968); and in cells doubly infected with *am12b* and *am5* (which produces coat protein), maturation protein synthesis late in infection is about one-half that seen in cells infected with *am12b* alone. Also, in contrast to *am12b*-infected cells, cells infected with a recently isolated temperature-sensitive mutant of the coat protein showed a normal rate of RNA synthesis and an increase of both RNA synthetase and maturation protein at high temperature (Nathans & Polmar, manuscript in preparation). Finally, in the cell-free synthesis of MS2 proteins, a protein product which corresponds in electrophoretic mobility to maturation protein is repressed by coat protein (Eggen & Nathans, 1969). Therefore, it is likely that the coat protein does regulate maturation protein synthesis.

The failure to observe equivalent derepression of maturation protein and synthetase in cells infected with the coat protein mutant *am12b* may be due to partial repressor activity of the coat protein fragment present in these cells, the fragment retaining more activity as a repressor for the maturation protein. Alternatively, some other masking of the maturation protein cistron may occur in *am12b*-infected cells as a result of the accumulation of unusual forms of phage RNA; as already mentioned, infection with this mutant leads to excess double-stranded RNA. Still another possibility is that the maturation protein itself, which is present in the infected cell complexed to phage RNA (Richelson & Nathans, 1967), may also repress its own synthesis.

As noted previously, the sequence of synthesis of phage proteins in infected cells is different from that seen in the cell-free synthesis of phage proteins directed by MS2 RNA (Ohtaka & Spiegelman, 1963; Eggen & Nathans, 1967). In the former case RNA synthetase appears first; in the latter case coat protein appears first, and a protein corresponding to synthetase appears last. Sequential synthesis *in vitro* is probably due to sequential translation of the polygenic viral RNA (Englehardt, Webster & Zinder, 1967; Capecchi, 1967), which may occur at a much slower rate than in the cell. The sequence in infected cells, on the other hand, may be related to the mode of entry of the infecting RNA strand, resulting in initial translation of the synthetase gene. Although the recent finding that the infecting RNA of a polar coat protein mutant is

not efficiently converted to a double-stranded form (Roberts & Gussin, 1967; Lodish, 1968) is evidence against this interpretation, further experiments will be required to exclude this explanation.

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